THE EFFECT OF HIGH PRESSURES ON BACTERIA

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The work recorded in this paper is the result of an attempt to study the mechanism of immunity and incidentally to test a theory regarding the failure of vaccine therapy as practiced at the present time.

During the past 8 or 10 years bacterial vaccines have been used extensively, but, on the whole, with very unsatisfactory results. Were it not for the excellent theoretical foundation on which vaccine therapy rests, its use would probably have been relegated to the past. Bacterial vaccines, as used today, judged by the practical results obtained, are of little or no value aside from typhoid vaccine as used prophylactically and the undisputed value of staphylococcic and B. acne vaccines, in certain types of cases.

Protective immunization experiments with killed cultures on laboratory animals have likewise not been crowned with marked success. Facts such as these may readily be gleaned from a survey of the literature. The conclusion would therefore seem justified that the dead organisms do not offer a suitable antigen for immunization processes.

During the past year while conducting some perfusion experiments on rabbits, we were much impressed by the rapidity with which bacteria were taken up by phagocytes. The particular experiment in question concerned the perfusion of the liver of rabbits with emulsions of staphylococci. By inserting cannulae in the portal vein and superior vena cava the liver could readily be perfused with Locke's fluid, containing large quantities of staphylococci in suspension. It was found that a fluid containing 9,000,000 organisms per cubic centimeter could be sterilized in a few minutes by being passed through this organ. The endothelial cells of the liver, on section, were found to be literally packed with bacteria following this operation. This observation suggested that vaccines injected into an individual for the purpose of

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immunization probably meet a similar fate. The phagocytes, whose function it is to seize any foreign material entering the body, failing to recognize the benevolent intention of the immunizer, probably take up the killed cultures with even more greed than the living bacteria. Metchnikoff[1] and his pupils have shown that the digestion of such incorporated particles by phagocytes may be very rapid, especially when it concerns dead bacteria.

It is a well recognized fact that an antigen which has undergone even partial digestion is no longer capable of stimulating antibody formation, and hence ceases to be an antigen. The natural deduction is therefore to assume that phagocytosis defeats, in a measure at least, attempts to immunize with killed bacterial cultures. Metchnikoff and his school would have us believe that the phagocytes are the source of antibody formation, but the evidence presented in support of this contention is far from conclusive.

If we now examine antigens, such as diphtheria and tetanus toxins, which are not in the form of particles, but in a diffused state and hence not so accessible to the phagocytes, there is little difficulty in attaining a high degree of immunity. An animal may likewise be given a few injections of foreign protein, such as egg white, with the result that a high titer of immune serum is formed. The exception to this rule would seem to be the immunization with tissue cells and red corpuscles in particular. A few injections of foreign red cells will usually give a serum with a very high lytic titer. This may, however, be explained by the fact that tissue cells are taken up by the so-called macrophages, which are large cells with a rigid nucleus, and which are slow to arrive on the field of action. It is not improbable that the antigenic principle is dissolved from the injected cells by the tissue fluids before phagocytosis takes place. Prompted by these considerations an attempt was made to extract the antigenic principle from bacteria in a form not so readily accessible to phagocytes in the hope that it might give vaccine therapy the much needed "raison d'être."

The conversion of bacteria from the solid or semisolid state to a state of diffusion is not an easy matter, owing to the firm outer membrane. Numerous investigators have subjected bacteria to various physical influences looking to this end, such as high pressure, shaking, freezing and grinding processes, but hitherto with little or no success.

E. Büchner[2] was the first to obtain definitely positive results by subjecting living cells to high pressure. By the use of a hydraulic press he was able to

[1] Metchnikoff: L'Immunite, 1901.

[2] Buchner, E.: Ber. d. deutsch. Chem. Ges., 1897, 30, 117.

separate zymase from the yeast cell. Later, Hans Büchner,[3] by using the same apparatus, subjected bacteria to a pressure of 500 atmospheres in an attempt to isolate ferments and endotoxins, but with rather disappointing results. Certes[4] found that a pressure of 350 to 500 atmospheres had no effect on putrefactive processes. Yeast cells under a pressure of 400 atmospheres were still able to ferment sugar. The same investigator found that the pathogenicity of anthrax bacilli was not impaired after having been subjected to a pressure of 600 atmospheres. D'Arsonval[5] and Charrin on the other hand found that CO_2 under a pressure of 50 atmospheres destroyed B. pyocyaneus in 2 hours. Their work was repeated by Sabruzes[6] and Bazin, Schaeffer[7] and Freudenreich, and Krause,[8] all of whom failed to confirm the findings of the French investigators. In summing up the results of high pressures on bacteria Gottschlich[9] concludes that they have little or no effect.

In our work many pieces of apparatus have been constructed, but only those with which definite results were obtained will be described. We first studied the effect of a direct load on bacteria.

For this purpose a piece of nickel-chrome-steel was obtained from one of the large steel companies, which also kindly furnished us with an analysis of the material. A hole ⅞ inch in diameter was bored through a piece of steel 5 inches by 3½ inches, and a piston, likewise of nickel-chrome-steel, was ground to fit the cylinder. One end of the cylinder was closed by a steel wedge. The cylinder, piston, and wedge were then tempered to withstand a pressure of 225,000 lbs. per square inch. The bacteria that were to be subjected to pressure were mixed with infusorial earth, after which they were placed in a leaden cup about 2 inches long and of a bore to fit the steel cylinder above described. The leaden cup, about two-thirds filled, was placed in the cylinder in an inverted position and forced to the bottom of the cylinder where it rested on a leaden disk which, in turn, rested on a steel wedge closing one end of the cylinder. The piston was then inserted and the apparatus placed in a testing machine such as is used by engineers in testing the strength of materials. The particular machine in question was capable of lifting a load of 200,000 lbs. The tests were usually inaugurated at 6 p. m. and terminated 14 hours later.

The following organisms have been studied in this manner: B. typhosus, B. coli, B. tuberculosis, B. proteus, B. subtilis, staphylococci, streptococci and pneumococci.

The pressures used ranged from 3,000-12,000 atmospheres. On termination of each experiment cultures were made on dextrose broth and, whenever possible, plates were made before and after the experiment. The general statement may be made that a pressure of 3,000 atmospheres was not sufficient to destroy any of the bacteria studied,

[3] Buchner, E.: München. med. Wchnschr., 1897, 44, 299.
[4] Certes: Compt. rend. Acad. de Sc., 1889, 99, 385.
[5] d'Arsonval & Charrin: Compt. rend. Soc. Biol., 1893, 467, 764.
[6] Sabrazés & Bazin: Koch's Jahresb. f. Gärungsorganism, 1893, 34.
[7] Schaffer & Freudenreich: Ann. d. Micrographie, 1890, 4, 502.
[8] Krause: Centralblatt f. Bakt., etc., 1902, O., 31, 673.
[9] Gotschlich: Kolle-Wassermann Handb. d. path. Mik., 2 Aufl., 3, 460.

while a pressure of 6,000 atmospheres for 14 hours was found to destroy all non-spore-forming organisms. The spores of B. subtilis were not regularly killed at pressures of 12,000 atmospheres. The bacteria would undoubtedly have been destroyed by these pressures in less time than that used; further studies are being undertaken to determine this point, however.

Having thus determined that it was possible to destroy bacteria by physical pressure, the question naturally arose as to the mechanism operative in this procedure. It was probably not a question of expressing the cytoplasm from the organisms, as was attempted by Büchner, inasmuch as the pressure exerted on the bacteria was uniform from all sides. The small amount of entrapped oxygen in the container could scarcely be responsible for the phenomenon. It was also felt that the element of heat development could be ruled out without further consideration. Two other factors were still to be considered: the direct effect of the pressure, and its sudden release. Which of these two was the important factor was, by the very nature of the experiment, difficult to determine, but it was not improbable that both factors played an important rôle in killing the bacteria. The interesting observation was made that bacteria subjected to high pressure were difficult to stain; as a rule only shadows could be discerned where it concerned gram-negative organisms, while the gram-positive ones usually lost their specific staining reaction.

It would seem probable from these experiments that the death of the bacteria was due either directly to the pressure or its sudden release or to a possible combination of both.

In an effort to analyze more fully some of the problems which had presented themselves during the process of these investigations, studies were inaugurated to determine the effect on various bacteria of gases under high pressure, such as CO_2, H, and N. An apparatus was constructed for this purpose which would enable us to subject bacteria to various gas pressures and suddenly release it. The apparatus used for these experiments may briefly be described as follows:

A steel tube, 8 inches in length and 1½ inches in diameter, lined with block tin, was attached to the desired gas tank by a suitable coupling. The opposite end of the apparatus was fitted to a brass head containing a small receiving chamber. The bacteria to be studied were placed in a sterile test tube resting on a coiled spring in the bottom of the tin-lined steel tube. The receiving chamber was provided with a valve, connected with a glass tubing which extended to the bottom of the test tube containing the bacteria. The apparatus was then attached to the gas tank and left for the desired time. When the

valve leading to the receiving chamber was opened, the pressure of the gas forced the bacterial suspension up the glass tube, through the valve into the receiving chamber. The fluid so driven into the receiving chamber could be collected from a small spout into a sterile test tube. It will thus be seen that the pressure was released suddenly as the fluid passed the valve. The valve was under such control as to permit the removal of the fluid drop by drop if desired. We are indebted to Mr. David Crowther, Mechanician and Instructor in Dental Mechanics, University of Minnesota, for valuable mechanical assistance. We are indebted also to Dr. J. F. McClendon for helpful suggestions.

It was found that CO_2 under a pressure of 50 atmospheres would destroy B. typhosus, B. coli, B. tuberculosis, B. pyocyaneus, staphylococci, streptococci, and pneumococci in a period of time ranging from 1½-2½ hours. It was interesting to observe that the gram-negative bacilli could be brought to a marked degree of disintegration, although disintegration of all the bacteria in suspension was never attained. The gram-positive cocci, on the other hand, suffered little morphologic change aside from slight irregularity in size, and often a tendency to lose their gram-positive character. The importance of suspending bacteria in distilled water instead of broth or physiologic salt solution was emphasized by the fact that when the latter were used very little disintegration of the organisms was noted. It was found that CO_2 of less than 40 atmospheres produced no effect whatever on the bacteria studied. Yeast cells were found to be unaffected by CO_2 after exposure to this gas for 48 hours. This may be due to the fact that yeast cells are normally active CO_2 producers, and their external membrane therefore probably offers little or no resistance to the CO_2 molecule. The osmotic equilibrium of these cells was probably not disturbed by the sudden change in the molecular concentration of the fluid. CO_2 likewise has no effect on the proteolytic ferments of B. pyocyaneus and B. proteus; these ferments were found to be as active after exposure to this gas at 67 atmospheres for 20 hours as were the controls kept at room temperature.

Having then found that bacteria could be destroyed by relatively low gas pressure, we were once more confronted with the problem as to the mechanism of the various physical and chemical forces involved in the experiment.

It seemed probable that the acidity of the CO_2 solution under the pressure used might be responsible for the killing of the organisms, although their disintegration was evidently due to the sudden expansion of the dissolved gas. Being unable to find any work dealing with the degree of acidity of CO_2 solutions under pressure, experiments to

determine the H ion concentration of this gas under various pressures were undertaken. The methods suitable for this particular experiment were, owing to its peculiar nature, limited. The colorimetric method seemed to be the most feasible. For this experiment a steel tube 4 inches long and 1 inch in diameter was lined with pure block tin. The ends of the tube were closed with glass lenses, supported further by heavy steel burrs. The indicator to be used was then placed in this apparatus which by means of a suitable coupling was attached to the CO_2 tank. Any change in the indicator could thus readily be determined. In order to be able to detect even a slight change in the indicator it was found necessary to construct a duplicate apparatus always containing the same indicator which could be used as a control. The P_H was found to be between 10-3 and 10-4, though probably nearer 4 than 3. It was found that the amount of pressure used had very little effect on the degree of acidity; in fact, experimentally, it was difficult to show any difference in the degree of acidity between 1 and 50 atmospheres' pressure, although theoretically the P_H of CO_2 at 50 atmospheres is 10-3.15 and at 1 atmosphere 10-3.6. Our failure to establish any difference in the degree of acidity of this gas under various pressures was probably an insufficient number of indicators of the proper range were used.

The next logical step in this series of experiments seemed to be to determine the effect of this degree of H ion concentration on the bacteria studied. Hydrochloric acid, acetic and phosphoric acids were prepared to a H ion concentration of 10-3.15, the maximum theoretical acidity of the CO_2 used. It was found that B. coli and the staphylococcus would live from 7-10 days in the hydrochloric and acetic acid of this acidity. The phosphoric acid (which is not buffered appreciably by proteins) was found to be more injurious to the organisms. However, both the staphylococcus and B. coli survived the action of the phosphoric acid for 48 hours. Our findings in this respect do not coincide fully with those of Michaelis,[10] who found that B. coli was destroyed in 48 hours in a solution of lactic acid at a P_H of 10-4. It is not improbable that various strains of the same organism may vary in regard to their resistance to acids.

These experiments seem to justify the conclusion that the acidity of the CO_2 is not an important factor in the destruction of the organism. It therefore seemed probable that the high molecular concen-

[10] Michaelis: Ztschr. f. Immunitätsforsch., 1912, O., 14, 170.

tration or the sudden change in the osmotic tension of the fluid, or possibly both, were the factors which destroyed the bacteria. CO_2 is very soluble in water, 0.8 of a volume being soluble for each atmospheric pressure. The maximal CO_2 pressure used in our experiments was 67 atmospheres, which, according to the law of the solubility of gases, would cause 52 volumes of CO_2 to go into solution. In order to determine the effect of this molecular tension on bacteria a solution of NaCl of corresponding concentration (7.2%) was prepared. Staphylococci and B. coli were suspended in this solution, which was acidified to a point of P_H 10-3.15, and cultures were taken hourly. It was found that these organisms were killed in about 15 hours. It does not follow, naturally, that, because the solution of CO_2 and sodium chlorid were of the same molecular concentration, their effect on bacteria would be identical. The effect of each on the membrane of the bacteria might be widely different; possibly the size of the molecule might be a vital factor, not to mention the possibility of the influence of incidental chemical reactions. Ignoring, for argument's sake, these unknown factors, and assuming the effect of the two solutions on bacteria to be much the same, we are led to the conclusion that the sudden release of the pressure, thereby lowering the osmotic tension of the fluid, is the vital factor in killing the bacteria, as it was found that bacteria were killed in from 1½-2½ hours in CO_2 solutions, whereas 15 hours were necessary to kill them in acidified salt solution of the same molecular tension. The 1½-2½ hours probably represents the time required for the gas to become dissolved in the water and to diffuse through the bacterial membranes.

The effect of hydrogen on bacteria was likewise studied. Hydrogen being only slightly soluble, it was not expected that its effect would be so marked as that of CO_2, its solubility being 0.031 volumes per atmosphere. Bacteria were placed in the apparatus described in the previous experiment and attached to a hydrogen tank under pressure of 120 atmospheres. It will thus be seen that only 2.2 volumes of hydrogen could be dissolved as compared with 52 volumes of CO_2. Sterile cultures were never obtained under the influence of hydrogen, although by plating the bacteria before and after treatment it was possible to demonstrate that from 10-40% of B. coli had been killed in 24 hours. There was evidence that many of the bacteria were broken up under the influence of hydrogen and this seemed in particular to be true of the tubercle bacilli. The most marked effect of

hydrogen on bacteria, however, was its tendency to affect their staining reaction. Gram-positive organisms would often become gram-negative, and even the acid-fast character of the tubercle bacilli was impaired.

A study was made of the effect of hydrogen on the pathogenicity of pneumococci; it was found, however, that the pathogencity of these organisms for white mice remained unaltered.

Nitrogen under a pressure of 120 atmospheres seemed to be entirely inert. The bacteria were not killed; neither did they present any morphologic change.

IMMUNIZATION EXPERIMENTS

As stated at the beginning, our object was to obtain the antigenic principle of bacteria in a diffused state in order to make it less accessible to the phagocytes, thereby hoping to obtain an antigen capable of producing a higher degree of immunity than is ordinarily possible with bacterial antigens. Typhoid bacilli which had been under orthostatic pressure of from 6,000-12,000 atmospheres were placed on a shaking machine for 10 hours, after which they were filtered through a Berkefeld filter and the filtrate injected into rabbits.

The question of a standard dosage at once became a serious problem. Bacteria which had been under pressure were difficult to standardize. The following technic was therefore adopted. Six 24-hour slant agar cultures were mixed with infusorial earth and placed under pressure for 14 hours. On removal from the pressure apparatus the infusorial-earth-bacterial mixture was taken up in 50 c c of distilled water and placed on the shaking machine and later filtered. Two c c of the filtrate were then injected into the marginal ear vein of rabbits at 5-day intervals. Ten days following the third injection the agglutinating titer of the blood serum was determined. It was not uncommon to find that the serum of rabbits thus treated would agglutinate typhoid bacilli in dilutions of 1 : 10,000. We rarely found the titer to be below 1 : 5,000. Rabbits treated with living typhoid bacilli in the same manner never yielded a serum of this agglutinating titer: from 1 : 100 to 1 : 500 being the average agglutinating titer of the serum of rabbits treated in this way.

Animals were likewise immunized with bacteria and bacterial filtrate of cultures killed by CO_2. Although these experiments are not yet terminated it may be said that bacteria killed by CO_2 give excellent results in the way of stimulating antibody formation, it being found that typhoid bacilli killed in this way gave an agglutinating serum of a

higher titer than when a corresponding amount of the living culture was used. The B. coli as well as other gram-negative bacilli, it was observed, became far more toxic after having been killed by CO_2 than the living cultures. This is probably due to the fact that large amounts of bacterial protein were liberated by the killing process.

A few attempts to immunize with the hydrogen-treated bacteria have been undertaken. In studying the complement-fixation reaction in tuberculosis excellent results were obtained with antigens which had been disintegrated by hydrogen.

RÉSUMÉ

It has been found that a direct pressure of 6,000 atmospheres kills non-spore-forming bacteria in 14 hours. A pressure of about 12,000 atmospheres for the same length of time is required to kill spores.

Non-spore-bearing bacteria are killed by CO_2 of 50 atmospheres pressure in about 1½ hours. Yeast cells withstand the action of CO_2 for more than 24 hours, probably because of their ability to transmit the CO_2 molecule through the membrane promptly.

The maximum acidity of solutions of CO_2 used in our experiments was P_H 10-3.15. It was shown that this degree of acidity was not a factor in killing the bacteria. A solution of sodium chlorid of a molecular concentration corresponding to a solution of CO_2 under 67 atmospheres and acidified to P_H 10-3.15 required 15 hours to render cultures of staphylococci and B. coli sterile. It was therefore concluded that the sudden change in the osmotic tension of the fluid in which the bacteria were suspended was the factor which destroyed the organisms.

Many of the gram-negative bacteria could be broken up by the sudden release of the CO_2 while the gram-positive bacteria, although killed, suffered little or no morphologic change.

Hydrogen killed from 10-40% of B. coli. This gas had no effect on the pathogenicity of pneumococci.

The acid-fast character of the B. tuberculosis was impaired by the action of hydrogen. Hydrogen-treated tubercle bacilli yielded a suitable antigen for the complement fixation reaction.

Nitrogen under a pressure of 120 atmospheres had no effect on bacteria.

Filtrate of typhoid bacteria which had been subjected to a direct load of 6,000 atmospheres for 14 hours was found to be far superior to the living culture as an immunizing antigen. Bacteria killed by CO_2 were likewise found to be excellent antigens.